Summary

In recent years, anthropology has been witnessing paradigm shift in the study of human evolution and human variation from genomic diversity to complex disorders and adopted molecular genetics approach using more sophisticated tools and techniques of DNA technology, which in turn has provided an unprecedented opportunity for researchers to dissect complex diseases and gene wise association studies to identify high risk patients and improve human health. Attention is also increasingly focused on elucidating genetic susceptibility/resistance to the diseases. A case control association analysis is often used to investigate whether certain variants in candidate genes are involved in disease susceptibility.

Throughout the history, malaria has proved to be a significant threat to human health and even today malaria continues to be a major global public health burden. Indian sub-continent is one of the major endemic regions besides Africa. Charles Alphonse Louis Laveran discovered the causative agent of malaria in 1880. Malaria is caused in humans by four species of single-celled *Plasmodium* protozoa parasites: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malaria*, with *P. falciparum* accounting for the majority of infections and being the most lethal. Transmission of the parasites is via the Anopheline mosquitoes, and is affected by climate and geography.

Plasmodium *falciparum malaria* remains a serious medical challenge. It affects over 200 million people and in Africa alone is responsible for over one million deaths among children each year. Cerebral malaria is a most severe and frequently fatal consequence of *falciparum* malaria in the young and non-immune age. Cerebral malaria causes acute symptoms including leaky blood vessels in the brain and cerebral odema. Anti-malarial chemotherapy initiated at the time of cerebral symptoms often fails to alter the fatal outcome, and mortality even with the best current therapies ranges from 10 to 50%. The pathological processes responsible for the cerebral manifestations are not well understood. Cerebral tissue obtained post-mortem reveals grossly congested cerebral vessels, occluded with infected red blood cells adherent to the endothelium, which leads to the subsequent encephalopathy secondary to cerebral anoxia. The vast majority of malaria-related deaths in India also are due to infection with Plasmodium *falciparum*. The outcome of the Plasmodium infection involves an intricate interaction among the parasite, the Anopheles mosquito, and the human host that results in a wide range of clinical manifestations ranging from mild febrile disease to cerebral malaria, acute respiratory distress, pulmonary oedema, renal failure, and severe anemia (Baird 1995; Sherman 1998). The question

is that why some individuals and other non-immune hosts die out of severe form of the disease while others develop an uncomplicated illness remains far from being understood.

Due to repeated exposure to infection, people living in malaria endemic areas gradually acquire mechanisms to limit the inflammatory response to the parasite that causes the acute febrile symptoms (clinical immunity) as well as mechanisms to kill parasites or inhibit parasite replication (antiparasite immunity). Children, who have yet to develop protective immune mechanisms, are thus at greater risk of clinical malaria, severe disease and death than adults. However, this is perhaps an oversimplified model and the degree of severity of the disease is multi-factorial. On one hand, parasite strains may differ in virulence (Skamene et al 1997), on the other, the influence of host genetic factors has been demonstrated in experimental models to play a role (Abel et al 1992; Hill 1996). Several genetic factors have been shown to control malaria disease blood infection levels and antimalarial immune responses in humans (Garcia et al 1996; Gilles and Warrell 1992).

WHO estimates that 207 million cases of malaria occurred globally in 2012 and 627 000 deaths. Most cases (80%) and deaths (90%) occurred in Africa, and most deaths (77%) were in children under 5 years of age (WHO 2013). In India, according to statistics of the National Vector Borne Disease Control Program (NVBDCP), approximately one million cases of malaria were reported during 2012, of which 50% cases of P. *falciparum* malaria. India contributed 13% in South East Asia region malaria cases (WHO 2013). P. *falciparum* in a subset of patients can lead to a diffuse encephalopathy known as cerebral malaria, a major contributor to malaria-associated mortality (Greenwood et al 1987). It shows the seriousness of the problem and global burden of disease thus it is necessary to provide genetic protection against malaria.

The main organs involved in malaria are liver, red blood cells and spleen. The major effect of malaria on peripheral blood is anaemia (Conard, 1969). This is mainly due to haemolysis however, other factors like ineffective erythropoeisis and splenic sequestration also play some role. It is particularly characteristic of *falciparum* malaria. The prevalence and degree of anaemia also depend on immune status of the patient, nutritional background and other complicating factors. Other abnormalities like thrombocytopaenia, leucopaenia and DIC also occur in patients of malaria. Leukocyte count may be normal but patients often have leucopaenia especially there is decrease in granulocyte count (Wernsdorfer, 1988).

A data-sharing community work to develop new tools to control malaria by integrating epidemiology with genome science. The Malaria Genomic Epidemiology Network (Malaria

GEN) is a multi-country collaborative effort to put in place the necessary infrastructure to conduct genome-wide and multi-centre association studies of resistance to malaria, in order to gain fundamental new insights into the effects of genetic variation on malaria susceptibility, and thereby on molecular mechanisms of protective immune responses and pathogenesis in endemic populations (Kwiatkowski 2005; Malaria GEN Consortium 2008). The idea that malaria has been acting as a major evolutionary force in recent human history (Kwiatkowski 2005) has been also the fertile ground for the development of tools to interrogate the human genome for signatures of positive selection (Tishkoff et al. 2001; Hamblin et al. 2002; Sabeti et al. 2002, Sabeti et al. 2006). Association findings of candidate-gene studies are now increasingly supported by evidence of selection at the locus. More importantly, signatures of malaria selection can be used with great value to direct the design and interpretation of association studies, now moving towards a genome-wide era (Mangano 2008).

A brief review of significant histological development in Plasmodium *falciparum* Malaria association studies:

Strong TNF promoter SNP association were first described in Plasmodium *falciaprum* malaria. Clinical studies had shown correlation between high TNF levels and severe malaria (Kwiatowaski et al. 1989; Kern et al. 1989) and the higher circulating levels of TNF cytokine correlated with disease severity and death (Kwiatowaski 1990; Kwiatowaski et al. 1989; Clark et a. 19990). Although mild to moderate amounts of TNF may be good for the host by suppressing and killing the parasite, excessive TNF alpha production is bad and may contribute to the pathology of severe infection and fatal out come.

First work on TNF polymorphism association study was fatal cerebral malaria is associated with high circulating levels of TNFA, McGuire et al. (1994) undertook a large case-control study in Gambian children. McGuire studied that homozygotes for the TNF2 allele (-308G-A) a variant of the TNFA gene promoter region, had a relative risk of seven fold for death or severe neurologic sequelae due to cerebral malaria. At same time the TNF2 allele is in linkage disequilibrium with several closer HLA alleles, and the study purposed that disease association was independent of HLA class I and class II variation and suggested that regulatory polymorphisms of cytokine genes can affect the outcome of severe infection. The maintenance of the TNF2 allele at a gene frequency of 0.16 in The Gambia implies that the increased risk of cerebral malaria in homozygotes.

TNF-α is a pro-inflammatory cytokine that has attracted particular interest because of its ambiguous activity in host defence and pathogenesis of cerebral malaria and other serious complications (Kwiatkowski 2000). High concentrations of TNF-α are related to the pathogenesis of symptoms associated with malaria, such as fever, and severe forms of infection, such as cerebral malaria (Kwiatkowski et al 1990; Karunaweera et al 1992). However, TNF-α has also been associated with the presence of potent antiparasitic activity, and persistent high levels of the cytokine lead to a rapid improvement in fever and a reduction in parasitemia (Mordm¨uller et al 1997; Depinay et al 2011). Genetic alterations in the TNF gene have been described in several studies with different populations in the world and sometimes with contradictory results. Population differences in susceptibility or resistance to malaria according to TNF SNPs may be a result of diverse evolutionary pressure between ethnicities, as well as different parasite strains and incidence of severe forms of disease. In Gambia, the SNPs TNF −308G>A (rs1800629) and TNF −238G>A (rs361525) were associated with an increased risk of cerebral malaria and severe malarial anaemia, respectively (McGuire et al 1994; Clark et al 2009). Studies in Gabon associated the TNF −308G>A polymorphism with a shorter interval to malaria reinfection and the TNF −238G>A polymorphism with protection against mild symptomatic malaria (Mombo et al 2003; Meyer et al 2002). In Sri Lanka, the TNF −308A allele was associated with severe malaria and other infections (Wattavidanage et al 1999). In another study in Mynamar, the TNFPD allele haplotype (−238G; −308G; −857T, rs1799724; −1031T, rs1799964) was associated with increased susceptibility to cerebral malaria because the transcription factor OCT-1 binds to TNF −857T in the TNFPD allele but not to TNF −857C in the TNFPA, B and C alleles and interacts with the pro-inflammatory NF-κB subunit transcription factor p65 at the adjacent binding site (Ubalee et al 2001). Other studies have shown no association between TNF gene polymorphisms and severe malaria in Kenya, Malawi, Mali, Tanzania, and Indonesia (Clark et al 2009; Stirnadel et al 1999; Randall et al 2010; Cabantous et al 2006). Tumor necrosis factor (TNF) is a proinflammatory multifunctional cytokine predominantly secreted by monocytes/macrophages that has effects on endothelial function, insulin resistance, coagulation and lipid metabolism. In the beginning, TNF was originally identified in mouse serum after injection with Mycobacterium bovis strain bacillus Calmette-Guerin (BCG) and endotoxin. Serum from such animals was cytotoxic or cytostatic to a number of mouse and human transformed cell lines and produced hemorrhagic necrosis and in some instances complete regression of certain transplanted tumors in mice (Shirai et al. 1985; Pennica et al. 1984).

TNF function during P. *falciparum* malaria infection has been described as both pathogenic and protective (Hensmann et al. 2001; Gimenez et al. 2003). At low levels, TNF is believed to increase parasite killing by subsequent release of cytokines and macrophage activation, whereas high TNF level has been associated with severe manifestations like acute respiratory distress and cerebral malaria. Because of its extensive role in malaria and reported activity against viral, bacterial and other parasitic infections TNF has been studied in malaria. In both in vivo and in vitro studies TNF have provided evidence that it is involved in inhibition of malaria parasite (Clark et al. 1987b; Taverne et al. 1987; Haidaris et al. 1983), indicating its ability for the potential immunogenetic marker and also a promising candidate for recombinant therapeutics for anti malarial drug development. In previous studies, TNF polymorphisms have been related to severe malaria. SNPs at position −1031,-857,-308,-238 and −863 in the promoter region of TNF gene exhibit differential associations to malaria and TNF production in different populations suggesting that individual TNF responses may be genetically determined (Hananantachai et al. 2007; McGuire et al. 1999; Higuchi et al. 1998; Hohjoh et al. 2001). TNF1304 within intron 3 was associated with variation in mild malaria and parasitaemia (Flori et al. 2005), and TNF-308A and TNF-238A allele have been associated with high anti-P. falciparum antibodies (Carpenter et al.,2009; Rihet et al.,2004).

In India Sinha et al (2008) studied and reported that the severity of plasmodium *falciparum* malaria has been correlated with TNF-enhancer and FcγRIIa gene polymorphism in Indian population and found that minor alleles of -1031 and -863 SNPs were associated with susceptibility to severe malaria. Sohail et al (2008) reported that TNF-α concentration in patients with and without fever were found to be significant ($p = 0.0001$, $p = 0.0004$, respectively). The genotypic distribution for −308 G/A and −1031 T/C positions were found non-significant, but it was clinically potent to observe statistically significant distribution of genotypes ($p = 0.032$) in patients with and without fever.

There is lack of information about severity of disease association (*falciparum* malaria) with TNF gene in Chhattisgarh. Therefore, realizing its importance and to fill this lacuna the present study was undertaken with the following aims and objectives:

Aims and Objectives:

- To understand the basis of genetic control of blood infection level in patients infected with Plasmodium *falciparum* malaria in tribal areas of Chhattisgarh in relation to the degree of severity of the disease.

- To assess the role of DNA polymorphism in TNF-α gene in relation to susceptibility of malaria caused by P. *falciparum.*
- To study the association of IL12 β cytokine gene polymorphism with the susceptibility of P. *falciparum* malaria in Chhattisgarh.
- To determine the association of hematological alternations in patients with Plasmodium *falciparum* malaria and severity of the disease from highly affected zones of Chhattisgarh.

Hypothesis:

- Null Hypothesis: The selected polymorphic markers on candidate gene region may not be associated with P. *falciparum* malaria and the higher frequency among the cases may just be because of chance.
- Alternative Hypothesis: The selective polymorphic markers on candidate gene region may be significantly associated with P. *falciparum* malaria and the higher frequency among the cases not is because of chance.

The effort related to the work was started with the visit of the hospitals in the identified endemic malaria zone (Malaria Control Society, Chhattisgarh). After 2-3 meetings with the authorities the work was given green signal by allowing the investigator to attend their OPD and pathology lab. The whole process was formalized after the study was cleared by institutional ethical committee on human research.

The study design is epidemiological case control study. In the case control studies mainly two types of controls are considered (Song et al 2004) -

1. Hospital based control: In this case chances of false positive findings increases as it completely rules out all the possibility of the person suffering from the disease.
2. Population based control: In this case, the normal individuals from the population are selected randomly, in accordance with the inclusion and exclusion criteria.

The diagnosis of the cases was done by the concerned medical doctor. An interview schedule was formulated and was pre-tested and modified after pilot survey in consultation with the research supervisor. The same was administered among both the cases and the controls by the investigator herself. Medical reports of the cases were verified and collected before collecting

biological samples with their individual informed consent. The population based controls were selected matched by age, sex and ethnicity of the cases as far as possible, from household survey of the same region.

The data were collected during 2009-2013 from 450 individuals of either sex. Of these 225 were cases from District Hospitals, Primary Health Centres, Community Health Centre and Private Hospitals of six malaria endemic districts namely Rajnandgaon, Jashpur, Raigarh, Kanker, Balrampur and Dantewada of Chhattisgarh state. Rest 225 were controls from the general population of the same area. All gave informed written consent prior to their participation in the study. Power of the sample size was calculated at statistical power of 80% (Lwanga and Lemeshow, 1991).

Beside socio-demographic data, about 3-5 ml biological sample (venous blood) was collected with the help of technician with individual informed consent of the subject in EDTA filled tubes for hematological & genetic analysis. Each tube was assigned a unique patient identification. Tubes were transported in icebox within 24 hours to the Human Genomics Laboratory of School of Studies in Anthropology, Pt. Ravishankar Shukla University, Raipur (C.G.) for further laboratory analysis. Haematological test were performed using a ERMA INC fully automatic Blood Cell Counter 210. Cell counter provided the data on the White Cell counts (WBC), Hemoglobin (Hb) level, Red blood cell counts (RBCs) and the Platelets count (PLAT) etc. Genomic DNA was isolated from whole blood following Miller et al. (1988). TNF-308, -238 and IL12β mutant genotypes (homozygous) were determined using a polymerase chain reaction (PCR) procedure, both genes were amplified simultaneously with and an internal control, in Gene Amp PCR system 9700 (Applied Biosystems, USA). The primer sequences and protocols were similar as described by Wilson et al. (1997) for TNF-308, d'Alphonso and Momigliano-Richiardi (1994) for TNF-238 and Gene speci international Inc. for IL12β. The polymorphic amplification products were analyzed by gel electrophoresis and visualized under UV light in Gel Doc Kodak 200 Imaging System. Genotype distributions were compared between groups using the χ^2 test. The group attributable risk was estimated by standard methods (Odds ratio). Statistical analyses were performed using SPSS 16.0 version and Microsoft Excel (Microsoft Office, 2007).

Analysis of household characteristics revealed that *Kachha* house type, mud wall, open drainage system and absence of toilet facility were higher in all three types of symptom bases malaria cases. Cases and controls living in *Kachha* type of house with open drainage system had

higher risk of malaria, which suggests that household characteristics do play important role in prevalence of malaria (Haque et al. 2011). Occupation wise distribution of cases showed that all types (mild, severe and cerebral) of patients were engaged in agriculture work. These findings indicate that the different caste communities living in very remote regions of rural area, close to deep forest and having agricultural occupation accounted for highest P. *falciparum* malaria cases, which suggest that poor living conditions, poor access to health services and exposure to disease causing environment significantly increases the incidence of malaria in them (Haque et al. 2009). This is further supported by the fact that the proximity of health services and market that sell medicated bed nets and anti malarial drugs to villages (Haque et al., 2011).

Various socio demographic predictions like age, sex acts income endemic zone, residence household characteristics etc. were compared for the odds probability in cases and controls. It revealed that males had more than one fold higher risk than females in cases than controls [OR = 1.135, 95% CI = .782-1.650]. With respect age, cases less than 18 years age and between 18-45 yrs. age had nearly 1.2 fold higher risk than control [OR 1.198, 95% CI= 0.593-2.418] and [OR = 1.187, 95% CI = 0.736-1.914]. It showed that age and sex lead a significant effect on the risk of disease. Male have outdoor moving behaviours. They have to work in the field & sometime they sleep outside the house at night whereas mostly female have to be inside the home for household work. They also better clothed than males. This is also one of the important factors for chance to mosquito bite. Dhingra et al (2010) reported that higher malaria mortality rate was not only found in children but also in later middle age(15-69), it is consistent with the age relations previously reported for malaria mortality in selected urban hospital in India (RGI: MCDSR 2000).

Household characteristics of controls were compared with the cases and revealed that cases living in kaccha houses were at 1.7 fold higher risk for the disease [OR =78 95% CI= 1.119 – 2.861], mud wall type of house showed significant ($p< 0.005$) risk association with the disease in cases than controls. Whole open drainage system showed more than 2 fold risk for the disease in cases [OR=2.431, 95% CI = 1.617 – 3.654]. One report suggested that mud roofs had significantly higher risk of getting P. *falciparum* infection compared to those living in iron-sheet roofed houses (Odds Ratio 2.6; 95% Confidence Interval, 1.4–4.7) (Yé et al. 2006). Open drainage provide a good environment for mosquito inbreeding.

The present study is gene-based association study with two candidate genes for malaria susceptibility and severity using three SNPs as genetic markers. This study demonstrated the nature of association in TNF genes and IL12 Beta gene with severity to P. *falciparum* malaria in

population of Chhattisgarh, India. Present study is based on the hypothesis that whether polymorphism at candidate gene affects the severity of P. *falciparum* malaria in cases and controls.

Findings in the present study show that mutation at position −308 and −238 of the TNF promoter do not significantly correlates with the severity of P. *falciparum* in cases of Chhattisgarh. Similar results were reported in Gabon, where there was absence of both homozygosis and association between TNF alpha-308 and malaria complications (Meyer, May et al. 2002) and in Thai, lack of association of TNF alpha-308 promoter polymorphism with disease severity (Hananantachai et al., 2001). This finding is not consistent with the results reported by several other studies in which TNF alpha-308 allele confers the risk for CM (Brinkman et al. 1995; Knight and Kwiatkowski 1999). In Gambia, children who were homozygote for the TNF alpha-308 allele had a sevenfold increased risk of CM or fatal outcome course and in Sri Lankans; TNF2 was associated with two to three time higher risk for severe malaria (McGuire et al. 1994; McGuire et al. 1999; Wattavidanage et al.1999). Polymorphisms in TNF gene promoter have been reported to be associated with symptoms and severity of P. *falciparum* malaria in different African and Asian populations (Sinha et al. 2008; Clark et al. 2009). The reason behind this might be that different populations have had different selective pressures placed upon them resulting in a number of susceptibility alleles across different populations.

IL-12 plays a major role in cell-mediated immunity against a variety of pathogens by rapid induction of IFN-γ production39. Levels of IL-12 has been seen both to increase and decrease in correlation to Plasmodium *falciparum* density and ratio of IL-12 to TGF-β and others anti-inflammatory was seen to correlate inversely with disease severity (Perkins et al. 2000; Chaisavaneeyakorn et al. 2003; Ong'echa et al. 2008). In experimental models, IL-12 administration decreased mortality in association with a reduction in peak parasitemia that was dependent on IFN-γ and partially dependent on nitric oxide (Mohan and Stevenson 1998; Eng et al. 1995). Polymorphism in genes encoding the subunits of IL-12 has been associated with malaria outcome. Genetic variants of IL-12 related gene protected Kenyan children from severe malaria anaemia.

IL12B gene polymorphisms were associated with symptoms and severity of P. *falciparum* malaria in studies from African and Asian populations (Driss et al 2011). In the present study polymorphism in this gene are associated with susceptibility and severity to malaria caused by P. *falciparum* and also positively associated with its clinical outcomes. The results of the preent

study suggested that the effects of IL12B on malaria disease outcomes most likely result from the long evolutionary history of P. *falciparum* parasite within the human population.

Hematological changes, which are the most common complications, play a significant role in the malaria complications. Abnormalities in hematological parameters have been reported to consistently companion which comprise anemia, thrombocytopenia, a typical lymphocytosis and infrequently disseminated intravascular coagulation (Facer 1994). Leucopenia, leucocytosis, Neutopenia, Neutrophilia, Eosinophilia and monocytosis also have been reported (Murphy and Oldfeild 1996). The pathogenesis of anemia in malaria is particularly complex, multi factorial and incompletely understood. It is thought to result from a combination of hemolysis of parasitized red blood cells; accelerated removal of both parasitized and innocently un-parasitized red blood cell, depressed as well as ineffective erythropoiesis with dys-erythropoietic changes and anemia of chronic disease (Clark and Chaudhri, 1988; Angus et al. 1999). Anemia and thrombocytopenia are common classical changes in patients with Plasmodium falciparum malaria (Phillips and Pasvol 1992). White Blood Cell changes are less impressive and there has been conflicting reports regarding these changes. In addition to these low RBC counts were also observed. Hematological examinations in cases and controls of Chhattisgarh showed similar findings for present study.

Maximum cases and controls had anemia, thrombocytopenia, low RBC and WBC counts. The findings of our study show significant mean differences in mild, severe and cerebral cases for these hematological changes.

Present study also discussed the role of TNF-α in P. *falciparum* malaria which has been implicated and may cause ineffective erythropoiesis. As hemolysis is known to be one of the major causes for P. *falciparum* malaria but it should not be the only reason behind significantly increasing the problem. Red cell morphology in malaria patients also influenced by their nutritional status i.e., patients could be iron deficient, folic acid or vitamin B12 deficient or they may had other blood related disorders, which aggravates the severity of the anaemia in cases of this study.

Future direction:

The present study included a large sample from remote tribal area. Since Chhattisgarh state is one of the major endemic regions for P. *falciparum* malaria in India inhabited by large number of tribal population groups, thus undertaking such study is on population of Chhattisgarh is of

paramount importance. However, it will be important to replicate such study in other populations, especially tribal populations, because they are genetically homogeneous compared to outbred urban populations, hence ideal for undertaking genetic association studies. Such studies along with functional genomic approach, will give insight to understand various pathways of controlling *falciparum* malaria infection and also give insight which pathway successfully control malaria parasite. In future, this type of study may help to assess the effects of ethnic differences, transmission intensity and other environmental factors on the interaction of TNF-alpha and IL12 genes with severity of malaria